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Embryotoxic and teratogenic profile of tretracycline at environmentally relevant concentrations on *Cyprinus carpio*

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HIGHLIGHTS

- Embryotoxicity and teratogenicity induced by tetracycline on *Cyprinus carpio* was evaluated.
- The teratogenic index of tetracycline was 3.4.
- Tetracycline was embryolethal and teratogenic for oocytes and embryos of common carp.
- The main tetracycline malformations were malformation in tail, modified chorda structure, pericardical edema, scoliosis.

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ABSTRACT

The objective of this work was to evaluate whether tetracycline (TC) in environmentally relevant concentrations was able to induce alterations to embryonic development and teratogenic effects in oocytes and embryos of *Cyprinus carpio*. For this purpose, an embryolethality study was conducted and the lethal concentration 50 (LC_{50}) and effective concentration 50 of malformations (EC_{50}) were calculated, and with these data the teratogenic index (TI) was determined. The main alterations to embryonic development and the teratogenic effects produced by TC on embryos of *C. carpio* were determined using the Kimmel and Hersem scale adapted for *Cyprinus carpio*. LC_{50} and EC_{50} were respectively 500.08 and 145.3 $\mu g\,L^{-1}$.TC was shown to be teratogenic with teratogenic index of 3.44, and the main malformations identified in concentrations of 90–900 $\mu g\,L^{-1}$ were malformation in tail, modified chorda structure, pericardical edema, scoliosis and malformations of the heart. A significant decrease in concentration-dependent in Kimmel and Hersem score was observed. The results allow us to conclude that TC at environmentally relevant concentrations is capable of inducing embryotoxic and teratogenic effects, generating risk in the integrity of the common carp *C. Carpio*.

1. Introduction

Antibiotics are a group of drugs that are widely used in the world to cure diseases in humans and animals. This group of compounds was designed with the purpose of inhibiting or killing microorganisms that can cause diseases (Liu et al., 2018(Carvalho and Santos, 2016; Larsson, 2014; Liu et al., 2018). According to their chemical structure, antibiotics can be categorized into: beta-lactams, quinolones, tetracyclines, aminoglycosides, macrolides and sufonamides (Chang et al., 2015; Daghrir and Drogui, 2013). Antibiotics as the most prescribed and used group of drugs can reach aquatic environments in their unchanged form or as metabolites through the urine or excrement of patients, in addition to hospital or industrial discharges (Ben et al., 2019; Danner et al., 2019; Kumar et al., 2019). Its presence in the environment has been associated with deleterious effects in the organisms that are found present in aquatic environments (Hao et al., 2019; Zhou et al., 2018).

As previously mentioned, one of the most commonly used chemical groups within antibiotics is tetracyclines, and in this

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category especially tetracycline (TC). This drug is mainly used for infections: skin (rosacea, acne), genitourinary (*Gonococcia*, syphilis), gastrointestinal (dysentery, cholera, amebiasis, gastric ulcer, periodontal infections), respiratory (faringoamigdalitis, bronchitis and some forms of atypical pneumonia), other infections (recurrent fever, Q fever, exanthematic typhus, endemic typhus, actinomycosis, brucellosis) [Lešnik et al., 2015]. The data on the use of this antibiotic are very varied, however it is estimated that each year the production of TC is thousands of tons (Michalova and Novotna, 2004). Xie et al. (2010) mentioned that tetracyclines occupy the second place worldwide in production and use, and the first in China; this may be due to the fact that within the group of antibiotics these are the cheapest and in addition to these they are widely used in veterinary use (Roberts et al., 2012). In reference to its occurrence, several studies have shown the presence of TC in aquatic environments. Chen et al. (2018), reported concentrations of TC from rivers in southern China from 9.1 to 300.7 ng L^{-1} and in sediments from 6.0 to 95.7 μg kg^{-1}. Tran et al. (2018), conducted a review of the average concentrations of TC in wastewater treatment plants, finding in Asia concentrations of 12, 340 and 1536 ng L^{-1}, in North America of 48,000 and 3600 ng L^{-1} and in Europe of 850 and 790 ng L^{-1}, respectively in influents and effluents of these treatment plants. Guerra et al. (2014) found average concentrations of 53 ng L^{-1} TC in influents of sewage treatment plants in Canada. Shimizu et al. (2013), reported concentrations below 1000 ng L^{-1} in five countries of the Asian continent as Vietnam, Philippines, India, Indonesia and Malaysia. Campagnolo et al. (2002), reported concentrations of 11–540 μg L^{-1} from surface water, groundwater and from liquefied waste from swine manure storage lagoon in United States of America (USA).

Referring to reports of toxic effects induced by the presence of TC in aquatic environments, there are some studies mentioned below. Up to now, most studies have focused on assessing the acute toxicity of this antibiotic. For example, in one study it was determined that the acute toxicity at 48 h for *Daphnia magna* was 617.2 mg L^{-1}, for *Danio rerio* at 96 h of 406 mgL^{-1} and for *Carassius auratus* at 96 h of 322.8 mg L^{-1} (Hui-zhu et al., 2008). In another investigation, it was observed that the highest growth inhibition rates for TC-induced in *Microcystis aeruginosa* and *Selenastrum capricornutum* were 0.09 and 2.2 mg L^{-1} of TC, respectively (Halling-Sørensen, 2000). Another study showed that TC significantly inhibits the growth rate of *Microcystis aeruginosa* at concentrations of 0.1, 0.2, 0.5 and 1.0 mg L^{-1} and showed a hormetic behavior at the concentration of 0.05 mg L^{-1} (Yang et al., 2013). Although TC has not been evaluated as an endocrine disruptor, it is known that oxytetracycline and chlortetracycline (10 mg L^{-1}) are capable of inducing vitellogenin production in *Oryzias latipes* (Ji et al., 2010; Kim, 2007). Likewise, chlortetracycline and oxytetracycline (13–87 mg L^{-1}) have the capacity to affect important aspects in reproduction, such as the hatching process, the number of young per female and the population growth rate of organisms such as *Vibrio fischeri*, *Daphnia magna, Moina macrocopa* and *Oryzias latipes* (Park and Choi, 2008). The aforementioned findings reflect the need for further studies on TC in order to establish its toxicity profile.

As previously mentioned, TC is able to inhibit the growth of aquatic organisms, which may be related to modifications in gene expression, alterations in the signaling of transcription factors and modifications in the cell cycle (Dennery, 2007) and this can lead to alterations of the embryonic development and teratogenic effects.

In this study, the possibility of TC being able to induce embryonic development alterations and teratogenic effects at environmentally relevant concentrations was proposed, for this aim we use C*yprinus carpio* as a model organism, since it has proven to be sensitive to emerging contaminants (González-González et al., 2014) in his oocyte and embryonic state (Luja-Mondragón et al., 2019).

2. Material and methods

2.1. Reagents

Tetracycline hydrochloride standard ((4*S*,4a*S*,5a*S*,6*S*,12a*R*)-4-(dimethylamino)-1,6,10,11,12*a*-pentahydroxy-6-methyl-3,12-dioxo-4,4*a*,5,5*a*-tetrahydrotetracene-2-carboxamide; hydrochloride) utilized in the study was purchased from Sigma-Aldrich (St. Louis, MO). The purity of the standard was ≥98%, $C_{22}H_{24}N_2O_8 \cdot HCl$, with a molecular weight of 480.90 (CAS number 66-75-5).

Likewise, all the reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

2.2. Procurement and viability oocyte analysis for embryotoxicity and teratogenicity tests

Obtaining *Cyprinus carpio* oocytes was by a natural fertilization. This process was carried out in the Tiacaque carp farm, in the State of Mexico. For this, four females and eight males of reproductive age were placed in a fertilization pond. The bottom of the fertilization pond was covered with casuarina branches so that the oocytes were deposited in order to facilitate their harvest. In embryotoxicity and teratogenicity studies only fertilized oocytes were used in the blastula period at 2 h post-fertilization. The oocytes in the blastula period were exposed to environmentally relevant concentrations of TC [concentrations that have been found in occurrence studies around the world considering surface water, influent and effluent from wastewater treatment plants, hospital effluents and groundwater] (Campagnolo et al., 2002; Daghrir and Drogui, 2013; Koikeb et al., 2004).

2.3. Exposure

In order to carry out the experiments of embryonic development alterations and teratogenic effects of TC, the guidelines established by the OECD were followed in their test guideline *Test No. 236: Fish Embryo Acute Toxicity (FET) Test*, 2013, with modifications for *Cyprinus carpio* carried out by Luja-Mondragón et al. (2019). The environmentally relevant concentrations of TC used in the study were 0, 90, 180, 270, 360, 450, 540, 630, 720, 810 and 900 μg L^{-1}, and a control system, drug free. The test systems consisted of 24-well microplates, to which a randomly selected fertilized oocyte was placed, to form batches of 20 oocytes for each TC concentration tested. The tests were performed in triplicate. The microplates were maintained for 96 h at a temperature of 24 ± 1 °C and during natural light-dark photoperiods in the laboratory. Observations were made at 12, 24, 48, 72 and 96 hpf, using the Zeiss program for Windows, and photographs of the malformations presented in each egg of common carp were taken.

2.3.1. Embryolethality profile evaluation

Systems similar to those used in section 2.3 were used, 60 oocytes were observed for each TC concentration tested using a stereoscopic microscope. Observations were made at 96 hpf, and lethality was considered when coagulated oocytes occurred or when no heartbeat was detected. Subsequently, the live and dead and malformed oocytes were quantified. With these data the lethal concentration 50 (LC_{50}) [concentration that caused 50% death in the oocytes], and effective concentration 50 of malformations (EC_{50}) [concentration that caused malformations in 50% of the oocytes]

were determined and a linear regression of maximum likelihood was performed. The LC_{50} and EC_{50} were calculated, as well as the 95% confidence limits. The tests were performed in triplicate.

2.3.2. Calculation of LC_{50}, EC_{50s} and teratogenic index (TI) for TC

The trimmed Spearman-Karber method was used to calculate the LC_{50} and EC_{50}, using US-EPA software ver 1.5. (Hamilton et al., 1977). The teratogenic index of TC was calculated using the relationship between LC_{50} and EC_{50} of malformations. If the value of teratogenic index was greater than 1, TC was considered as teratogenic and if its value was less than 1 as embryolethal (Weigt et al., 2011).

2.3.3. Evaluation of the TC embryotoxic and teratogenic profile

Systems similar to those used in section 2.3 of material and methods were used. The embryos of *Cyprinus carpio* were observed under a stereomicroscope at 12, 24, 48, 72 and 96 h. To assess embryonic development disorders, the score established by Kimmel et al. (1995) and Hermsen et al. (2011) with modifications for *Cyprinus carpio* by Luja-Mondragón et al. (2019) was used.

Teratogenic malformations induced by TC were also identified with the same scale. The data obtained were used to determine the frequency histogram and record the main malformations induced by the study drug: delay in the hatching process, hypopigmentation, hemorrhaging in the head, hemorrhaging in the tail, hemorrhaging in the yolk, miscellaneous; severe malformations, modified chorda structure, malfortation of the head, malformation of the heart, malformation of tail, pericardial edema, scoliosis, yolk deformation and yolk edema.

2.4. Test validity and reliability and statistic analysis

In order to assure validity and reliability of the results, egg batches were only used if the fertilization rate was $\geq$90%. It is important to mention that the test is considered valid only if the controls showed no more than 10% of teratogenic effects at 96 hpf.

IBM SPSS Statistics 25 was employed to perform the statistical analysis of the data. One-way ANOVA followed by post hoc multicomparison with the Bonferroni's test was used to analyze homogeneous data of the continuous variables. Kruskal–Wallis test was used to analyze non-homogeneous data. The frequency of abnormal oocytes or embryos was evaluated with Fisher's exact test ($p < 0.05$).

3. Results

3.1. Embryolethality and teratogenicity at 96 h data induced by tetracycline (TC)

Table 1 shows the mortality and malformation data induced by environmentally relevant concentrations of TC. As can be seen, the concentration that generated the death of 50% of the oocytes and embryos exposed was 500.08 $\mu g\,L^{-1}$ with confidence intervals to 95% of [426.578–598.341], which has been identified in aquatic environment (Campagnolo et al., 2002). Likewise, the concentration that generated 50% of malformations in oocytes and embryos exposed was 145.3 $\mu g\,L^{-1}$ with confidence intervals to 95% of [92.709–192.325], more than three times lower than the LC_{50}. When calculating the value of the ratio of the LC_{50}/EC_{50} which is the teratogenic index, a value of 3.44 was obtained, so the TC is considered as a teratogenic drug (Weigt et al., 2011).

Fig. 1 shows the percentage of normal, with teratogenic alterations and dead oocytes of *Cyprinus carpio* in each TC concentration used in the experiment The teratogenic alterations remained constant at the first concentrations of TC 90 at 450 $\mu g\,L^{-1}$ (33–35%), reaching a maximum at the highest concentrations of TC 540 at 900 $\mu g\,L^{-1}$ (45%). The percentage of normal oocytes was decreasing directly proportional to the increase in concentrations (reaching 20% in the highest concentration). These data are consistent with Fig. 2, where we can observe that at the highest concentrations of TC a higher percentage of teratogenic alterations were observed.

3.2. Main teratogenic alterations and frequency of the same by exposure to TC in environmentally relevant concentrations

In Fig, 2 the frequency histogram of the teratogenic alterations

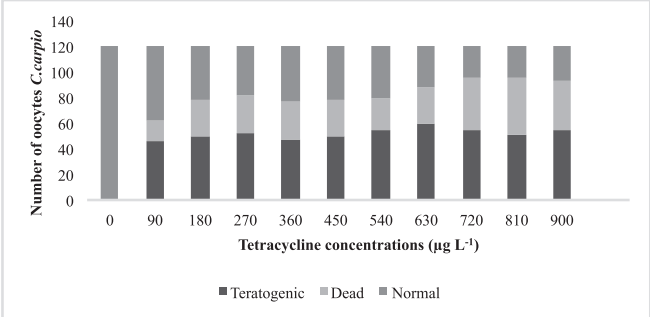


Fig. 1. Percentage of normal, teratogenic and dead oocytes or embryos of *C. carpio* exposed to the different TC concentrations.

Table 1
Mortality and malformation data in oocytes and embryos of *C. carpio* exposed to TC.

TC concentration ($\mu g\,L^{-1}$)	Number of embryos exposed	Mortality (%)	Malformations (%)
0	120	0	0
90	120	28.3	43.3
180	120	31.7	56.7
270	120	34.2	60
360	120	35.8	60.8
450	120	44.2	62.5
540	120	50.84	65
630	120	54.2	69.2
720	120	59.2	72.5
810	120	60.84	75.83
900	120	66.7	80.8
		$LC_{50} = 500.083$	$EC_{50} = 145.30$
		CI = [426.578–598.341]	CI = [92.709–192.325]
		Teratogenic index	
		3.442	

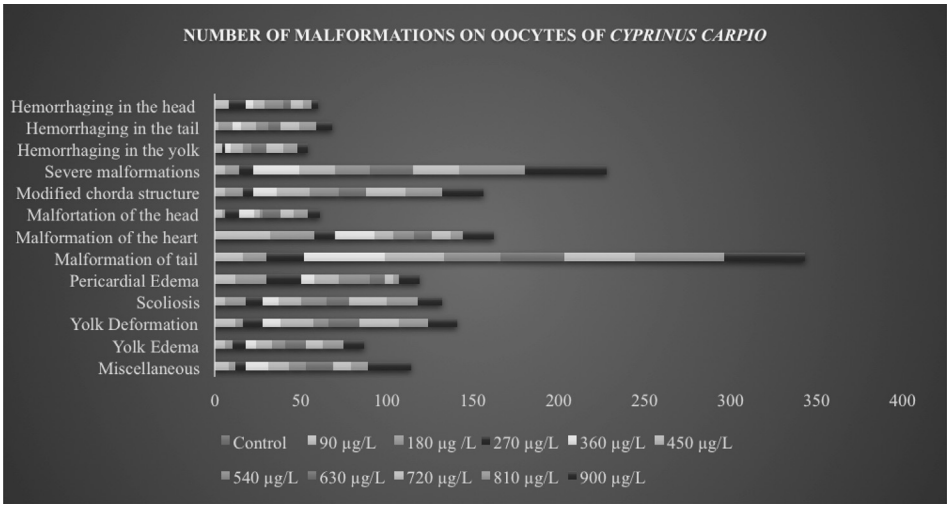


Fig. 2. Teratogenic malformations induced by exposure of oocytes and embryos of *C. carpio* to different TC concentrations.

presented in the different concentrations of TC is presented as the main malformations identified were: malformations in tail, malformation of the heart, modified structure chorda, scoliosis, yolk deformation, and other severe malformations that put at risk the survival of oocytes and embryos of *Cyprinus carpio.*

In Fig. 3, the different malfomations that the oocytes and embryos of *Cyprinus carpio* showed, by exposure to the different concentrations of TC and to the different exposure times. The control images represent the normal development of the oocyte, until it hatches at the different hpf. At 12 h, eye development was observed, at 24 h the somites formation, at 48 h, heartbeat, oocyte movement, pigmentation of the head and body were observed, at 72 h the hatching was observed, and development of the pectoral fin and at 96 h larva movements, protruding mouth, among other developmental characteristics. Subsequently, the embryonic development of *Cyprinus carpio* exposed to the different concentrations of TC (90–900 $\mu g\,L^{-1}$) was analyzed by comparison with the control group. The alterations to the embryonic development could be evidenced from 24 h by exposure to TC from the lowest concentration 90 $\mu g\,L^{-1}$ (yolk deformation) and up to 900 $\mu g\,L^{-1}$ (malformation of tail, scoliosis, modified chorda structure and pericardial edema). At 48 h the same malformations were observed at the highest concentration 900 $\mu g\,L^{-1}$ as at 24 h, in addition to heart malformations. As can be seen at 72 and 96 h, the most severe malformations occurred, in many cases there was a delay in hatching, and in these times there were severe cases of scoliosis, alterations of the notochord, pericardial edema, and malformations of tail. Although the data is not presented, many of the embryos died after 96 hpf.

Fig. 4 shows the results of the score obtained in each of the proven concentrations of TC at the different exposure times. The highest score obtained according to the Hersem scale modified by Luja-Mondragón et al. (2019) was in the control system, since oocyte development was normal. As the TC concentrations increased, the score decreased due to all developmental alterations and teratogenic effects observed ($p < 0.05$). All decreases were statistically significant with respect to the control score and at all exposure times.

4. Discussion

The results obtained in this investigation clearly demonstrate the embryotoxic profile and teratogenic potential of TC at environmentally relevant concentrations. In this study we decided to work with these concentrations, since these are present in bodies of water and these are the reflection of the presence of this antibiotic in the world (Campagnolo et al., 2002). Tetracycline has generally been shown to remain stable at pH greater than 6.5, however in acidic conditions pH 2.0–6.0 this antibiotic can be degraded and the main degradation products are 4-*epi*-tetracycline, anhydro-tetracycline and *iso*-tetracyclines (Kühne et al., 2000). In this experiment we monitored the temperature and pH and it remained constant at approximately 8.0, so we can attribute the effects to the TC and not to the degradation products.

A study conducted by our research group previously (Gómez-Oliván et al. 2014; send for publication) using *Cyprinus carpio* as a bioindicator, showed that biomarkers of cellular oxidation increased between 50 and 75% and cellular antioxidation decreased by 85% due to TC exposure, demonstrating that this drug is capable of producing oxidative stress in common carp.

These antecedents, promote the conduct of the present investigation and identify if the TC at environmentally relevant concentrations was able to produce alterations to embryonic development and teratogenic effects using oocytes or embryos of a species of commercial interest such as *Cyprinus carpio.* The results of LC showed that at 500.083 $\mu g\,L^{-1}$ the death of 50% of the oocytes was observed. In other studies such as the one established by Huizhu et al. (2008), it was shown that the TC showed an LC_{50}-96 h of 406.0 $mg\,L^{-1}$ using *Danio rerio* as a bioindicator. In another study Isidori et al. (2005) showed that the LC_{50} of tetracycline at 30 min was 60.5 $mg\,L^{-1}$ in *Vibrio fischeri.* According to these results, *C. carpio* oocytes were more sensitive to TC than *D. rerio* and *V fischeri* in youth states.

When calculating the value of the teratogenic index, we find a value of 3.44 which implies that the TC is highly teratogenic. We could verify that by establishing the histogram of malformations by identifying effects such as malformations in tail, malformation of the heart, modified structure chorda, scoliosis, yolk deformation, and other severe malformation (shown in Fig. 3).

The results of embryotoxicity and teratogenicity obtained in this study could be explained by the reactive oxygen species generated by exposure to TC (previously demonstrated in the lab). Also, Yang et al. (2013), showed an increase in the concentration of malondialdehyde (MDA) from 19% and up to 75% in TC concentrations ranging from 50 to 1000 $\mu g\,L^{-1}$ and increases of superoxide dismutase (SOD) activity of 52–173% in the same concentrations on

Fig. 3. Malformations induced by exposure to TC at 12, 24, 48, 72 and 96 h at the following concentrations: 0, 90, 180, 270, 360, 450, 540, 630, 720 and 900 µg L^{-1} on oocytes or embryos of *C. carpio*.

species such as *Microcystis aeruginosa* and *Selenastrum capricornutum*. MDA and SOD are considered good biomarkers of damage due to the presence of free radicals (reactive oxygen species). Also, Zhang et al. (2015) demonstrated that tetracycline 20 $\mu g\,L^{-1}$ was able to induce oxidative stress and alter the development of embryos of *Danio rerio*. The malformations observed by exposure to TC were hatching delay, shorter body length, increased yolk sac area and uninflated swim bladder. Besides, Nunes et al. (2015), showed that at TC concentrations of 5, 50 and 500 $ng\,L^{-1}$ there were alterations in the levels of TBARs, GST and CAT, as well as neurological alterations and histopathological effects on gills and liver of *Gambusia holbrooki*.

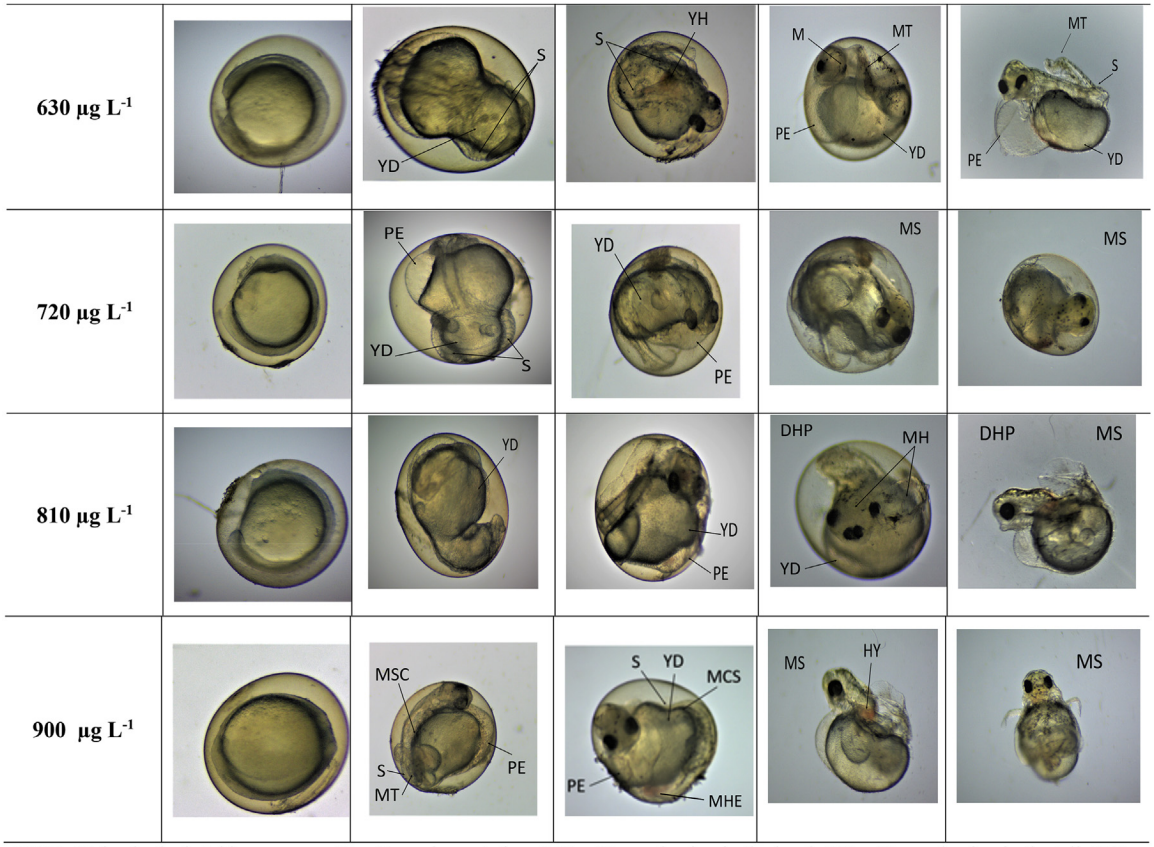
It is proven that exposure to environmental pollutants such as TC can significantly increase cortisol levels in fish by inducing ROS and generating oxidative stress (de Freitas Souza et al., 2019; Espinoza et al., 2017; Leong et al., 2009). Studies by Zhang et al. (2015), have shown that TC is capable of generating up-regulation of ROS genes (SOD and CAT) and overproduction of ROS in zebrafish embryos at 96 hpf.

The role of ROS in the induction of embryotoxicity and teratogenicity has been widely demonstrated. The increase in ROS is related to changes in cell metabolism, changes in mitochondrial kinetics and the bioactivation of chemicals to more reactive metabolites. Changes in ROS regulation can lead to abnormal embryogenic processes (Hansen et al., 2018; Veith, 2018; Wible and Bratton, 2018). It is well known that when there is an imbalance in redox processes, developmental alterations, structural malformations and neurobehavioral deficiencies can be generated (Cao et al., 2019; Souders et al., 2018).

As mentioned above, in addition to the fact that TC has been identified as an oxidative stress inducer, it should be considered that the early stages of embryonic development are very susceptible to oxidative damage, due to aerobic metabolism overload related to energy demand in the growth process, in addition to the conditions that lead to the high production of free radicals, the high levels of iron levels not bound to proteins (NPBI), and the lack of maturity of antioxidant systems (Petitjean et al., 2019).

Another situation that may be related to the increase in ROS is the biotransformation of tetracycline. (Zhang et al., 2015), showed that by exposure to TC in concentrations of 20–200 $\mu g\ L^{-1}$ a significant increase in CYP1A (isoenzyme responsible for the biotransformation of this antibiotic) was observed in zebrafish. It is important to note that, (Stegeman and Livingstone, 1998), characterized different families of cytochrome P450 genes in *Cyprinus carpio*, these families are: CYP1, CYP2, CYP3, CYP4, CYP11, CYP17 and CYP19. Therefore, the ROS responsible for the embryotoxic and teratogenic profile of the TC, may be derived from the biotransformation of the antibiotic or by the increase in cortisol levels in *C. Carpio*.

Of the malformations identified in the oocytes of *Cyprinus*



DHP= Delay in the hatching process**; H =** Hypopigmentation**; HH** = Hemorrhaging in the head; **HT** = Hemorrhaging in the tail; **HY =** Hemorrhaging in the yolk**; M** = Miscellaneous; **MS**= severe malformations; **MCS** =Modified chorda structure; **MH** = Malfortation of the head; **MHE** =Malformation of the heart; **MT** = Malformation of tail; **PE =** Pericardial Edema**; S** = Scoliosis; **YD** =Yolk Deformation; **YSE** = Yolk Edema

Fig. 3. (*continued*).

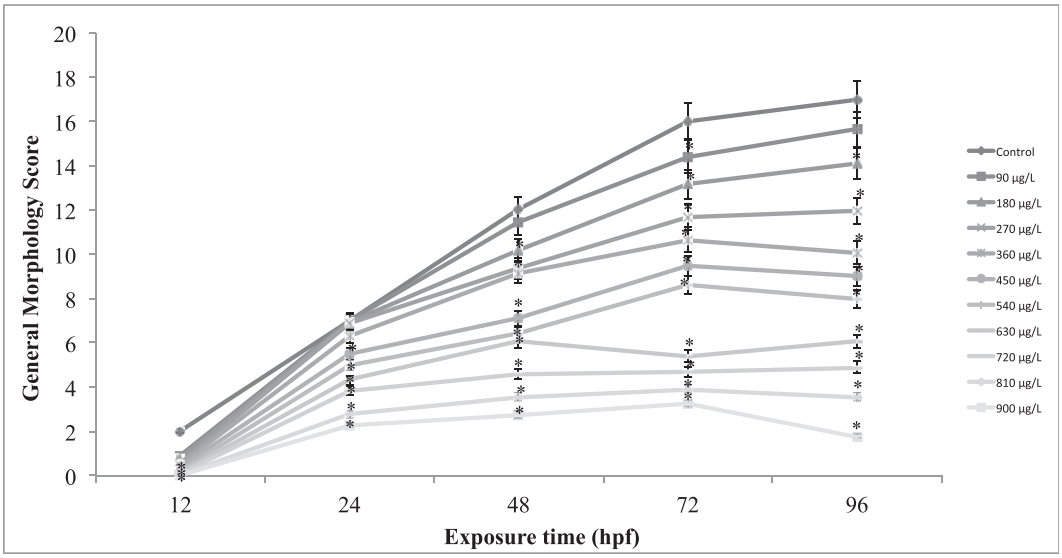


Fig. 4. Exposure time-response curve of the general morphology score of the TC concentrations on oocytes or embryos of *C. carpio.*

carpio, one that was of special interest was the deformation of the yolk sac. This structure is very important, in the fish since it plays a very important role during the early stage of development, because it is the only source of nutrition for embryos, and its physical size will decrease along with embryonic development (Shi and Zhou, 2010). Our results showed that exposure to the different concentrations of TC showed deformation of the yolk sac, which due to its functions resulted in other malformations found in the oocytes and embryos of the common carp. Our study also evidenced in an important way that the TC, was able to induce delay in the process of hatching in *C. carpio*. In some cases the hatching occurred until 96 hpf and in some cases the oocytes did not hatch, when normally the hatching in carp *C. carpio* occurs at 72 hpf. Osman et al. (2007), report that a late hatching process may be due to delayed development or the inability of embryos to break the chorion. Also, these alterations could be due to the inhibition of important enzymes in the hatching process such as chorionase (Haendel et al., 2004), by the presence of ROS, or because of the oxidative damage that ROS generate in the chorion of *C carpio* oocyte. In addition, as in the hatching process the oxygen requirements are greater in the oocytes, the imbalance in the redox status of the carp can cause developmental alterations.

Fig. 4 Clearly summarizes how TC is capable of altering the embryonic development of oocytes and embryos of *C. carpio* time-dependent. It is also observed, as the highest concentrations used in this study are capable of generating malformations and teratogenic effects that put the risk of common carp.

5. Conclusions

The data obtained in this study showed a teratogenic index of 3.44, showing that TC is an antibiotic that is capable of generating embryotoxicity and teratogenicity. The main malformations identified by exposure to TC in environmentally relevant concentrations were malformation in tail, modified chorda structure, pericardical edema, scoliosis and malformations of the heart. The alterations to embryonic development and teratogenic effects were time and concentration-dependent. The results allow us to conclude that TC at environmentally relevant concentrations is capable of inducing embryotoxic and teratogenic effects, generating risk in the integrity of the common carp *C. Carpio.*

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